Effect of P2 receptor blockade with pyridoxine on sympathetic response to exercise pressor reflex in humans

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Non-technical summary During exercise, sympathetic nervous system activity increases and this contributes to an increase in blood pressure (i.e. exercise pressor reflex). Although animal studies suggest that purinergic P2 receptors on thin fibre sensory nerves are stimulated and evoke this reflex, human data are lacking. In this study, young healthy volunteers performed fatiguing isometric handgrip before and after a local infusion of pyridoxine (i.e. vitamin B_6) into the 'isolated' circulation of the human forearm. Pyridoxine is converted into a P2-purinoceptor antagonist. Muscle sympathetic nerve activity and blood pressure responses to fatiguing handgrip and post-exercise circulatory occlusion were significantly less after pyridoxine than they were before. These effects were not observed after infusion of saline. These data suggest that P2 receptors contribute to the exercise pressor reflex in humans.

Abstract Animal studies suggest that ATP plays a role in evoking the muscle reflex via stimulating purinergic P2 receptors on sensory neurons. However, there are no human data regarding the role ATP and P2 receptors may play in evoking the exercise pressor reflex. We hypothesized that P2 receptor blockade in humans would attenuate the exercise pressor response. Blood pressure (Finometer), heart rate and muscle sympathetic nerve activity (MSNA; peroneal nerve) were assessed during fatiguing isometric handgrip, post-exercise circulatory occlusion (PECO), and passive muscle stretch during PECO in 10 young healthy volunteers. The protocol was performed before and after local infusion of pyridoxine hydrochloride (i.e. vitamin B_6) in saline via Bier block. Pyridoxine is converted into pyridoxal-5-phosphate, a P2-purinoceptor antagonist. In the second experiment, the same amount of saline was infused via the same procedure. After pyridoxine, the MSNA responses to fatiguing handgrip ($\Delta 349 \pm 70$ *vs.* $\Delta 556 \pm 92$), PECO ($\Delta 285 \pm 37$ *vs.* $\Delta 532 \pm 115$) and PECO + passive stretch ($\Delta 368 \pm 66$ *vs.* $\Delta 641 \pm 128$ units min^{-1}, all $P < 0.05$) were all significantly less than those before pyridoxine. The blood pressure responses were also significantly (all $P < 0.05$) less than those before pyridoxine. Infusion of saline (as opposed to pyridoxine) had no effect on the MSNA and blood pressure responses. These data are consistent with the concept that P2 receptors contribute to the exercise pressor reflex in humans.

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Abbreviations CPT, cold pressor test; EOW, extension of wrist; MAP, mean arterial pressure; MSNA, muscle sympathetic nerve activity; MVC, maximal voluntary contraction; PECO, post-exercise circulatory occlusion; PLP, pyridoxal-5-phosphate.

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Introduction

Exercise activates the sympathetic nervous system, and this contributes to an increase in heart rate, cardiac output, vascular resistance and blood pressure (Alam & Smirk, 1937; Mitchell & Wildenthal, 1974; Sinoway *et al.* 1989; Rowell, 1993). In addition to central mechanisms (i.e. central command) (Goodwin *et al.* 1972; Mitchell & Wildenthal, 1974; Vissing *et al.* 1991), it is believed that inputs from mechanically and chemically sensitive afferents from the exercising muscles are primarily responsible for this exercise pressor reflex (Mark *et al.* 1985; Seals, 1989; Sinoway *et al.* 1989). Group III and IV afferent fibres in muscles are suggested to be involved in this exercise pressor reflex (McCloskey & Mitchell, 1972; Waldrop *et al.* 1984). A number of substances are potential muscle afferent stimulants (Kaufman & Forster, 1996).

Previous studies have shown that muscle interstitial ATP concentrations increase with muscle contraction in animals (Li *et al.* 2003) and humans (Hellsten *et al.* 1998; Mortensen *et al.* 2009). Mechanical stimulation of muscle *per se* is a sufficient stimulus to raise interstitial ATP because muscle stretch in the absence of contraction raises ATP in the rats (Li *et al.* 2005). ATP is also co-released from sympathetic nerve terminals with noradrenaline (norepinephrine) (von Kügelgen & Starke, 1991; Vizi *et al.* 1992). Therefore, the mechanical stimulation of skeletal muscle as well as increases in sympathetic nerve activity during exercise could cause the release of ATP into muscle interstitium, where the free nerve endings of group III and IV muscle afferents reside. Importantly, animal studies have shown that ATP stimulates and sensitizes these thin fibre muscle afferents and contributes to the exercise pressor reflex (Hanna *et al.* 2002; Li & Sinoway, 2002). Receptors stimulated by ATP are termed P2 receptors, and are either ligand-gated ion channels (P2X) or G protein-coupled P2Y receptors, respectively (Ralevic & Burnstock, 1998). Animal studies have suggested that activation of P2 receptors, which are sensitized and stimulated by muscle contraction as well as muscle stretch, evokes a pressor response (Li & Sinoway, 2002; Hanna & Kaufman, 2003; Kindig *et al.* 2007*a*). Moreover, P2 antagonists attenuate responses of group III and IV afferents to static contraction and tendon stretch (Kindig *et al.* 2006), and the responses of group IV afferents to post-contraction occlusion in cats (Kindig *et al.* 2007*b*). This type of purinergic receptor in the muscle of humans may play an important role in mediating the magnitude of the autonomic adjustment seen with exercise. However, to date no data have been gathered to address this issue. Interestingly, pyridoxine hydrochloride (i.e. vitamin B_6) can be safely given to humans and is converted into pyridoxal-5-phosphate (PLP) (Solomon & Hillman, 1979; Jansonius, 1998), a P2-purinoceptor antagonist (Khakh *et al.* 1995; Ralevic & Burnstock, 1998; Millart *et al.* 2009).

Therefore, the purpose of the present human study was to examine the effects of P2 receptor blockade on sympathetic activation during exercise. We hypothesized that P2 receptor blockade in exercising muscle would attenuate the responses of muscle sympathetic nerve activity (MSNA) and blood pressure to isometric handgrip exercise and post-exercise circulatory occlusion (PECO). P2 receptor blockade in the exercising forearm was accomplished by infusion of pyridoxine hydrochloride (vitamin B_6) in saline via Bier block.

Methods

Subjects

Ten healthy subjects (9 male, 1 female, age: 25 ± 1 ($\pm$ S.E.M.) years; height: 177 ± 3 cm, weight: 74 ± 3 kg) participated in the study. All subjects were normotensive (supine blood pressures $<$140/90 mmHg), and no-one was taking any medication. Subjects refrained from caffeine, alcohol and exercise 24 h prior to the study. The experimental protocol was approved by the Institutional Review Board of the Milton S. Hershey Medical Center and conformed with the *Declaration of Helsinki*. Each subject had the purposes and risks of the protocol explained to them before written informed consent was obtained.

Measurements

Beat-by-beat blood pressure was recorded from a finger (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands) with resting values verified by auscultation of the brachial artery (Dinamap, Critikon, Tampa, FL, USA). Heart rate was monitored from the electrocardiogram (Cardicap®5, Datex-Ohmeda, GE Healthcare, NJ, USA). Respiratory frequency was monitored using piezoelectric pneumography. Multifibre recordings of MSNA were obtained with a tungsten microelectrode inserted in the peroneal nerve of a leg. A reference electrode was placed subcutaneously 2–3 cm from the recording electrode. The recording electrode was adjusted until a site was found in which muscle sympathetic bursts were clearly identified using previously established criteria (Vallbo *et al.* 1979). The nerve signal was amplified, band-pass filtered with a bandwidth of 500–5000 Hz, and integrated with a time constant of 0.1 s (Iowa Bioengineering, Iowa City, IA, USA). The nerve signal was also routed to a loudspeaker and a computer for monitoring throughout the study. Heart rate, blood pressure, MSNA and respiratory excursions were recorded throughout the studies. Passive stretch and handgrip forces were measured with transducers. Forearm volume was assessed by water displacement.

Venous samples were collected at the antecubital fossa of the exercising arm. Plasma PLP was used as an index of concentration of P2-purinoceptor antagonism. PLP levels were quantified by HPLC (high performance liquid chromatography) with fluorescence detection (Bisp *et al.* 2002).

Experimental design. All subjects were tested in the supine position. An intravenous catheter was inserted in the antecubital fossa of the non-dominant arm. The maximal voluntary contraction (MVC) of the non-dominant hand was tested during each visit. To ensure that the strength of the passive stretch was as vigorous as possible without evoking pain, the stretch strength for each subject was tested before the study. A specifically designed brace with a joint at the wrist was used to support the subject's forearm and hand. The hand was flexed in the dorsal direction (the extension of wrist, EOW) as the force was measured with a digital force gauge (IMADA, DPS-220, Northbrook, IL, USA). During EOW, the position of the forearm and wrist remained fixed. The maximal force used to stretch the muscles without inducing pain was obtained during the first visit, and was used for all stretch protocols performed on the two study days. No subjects complained of pain with EOW.

Pre-pyridoxine trial. The experimental protocols are illustrated in Fig. 1. After instrumentation, 6 min baseline measurements of heart rate, blood pressure, MSNA and respiratory excursion were collected with the subject in the resting condition. A baseline blood sample was obtained. Each subject then performed static isometric handgrip at 30% MVC to fatigue followed by 4 min of PECO. A visual force indicator was used so that subjects could maintain the force necessary for 30% MVC. During grip, subjects were asked to report their perceived level of effort using the Borg scale of 6–20 (Borg, 1998). The determination of fatigue rested with: (1) the inability of the volunteer to maintain the desired force production; and (2) the assessment of the volunteers that the work was 'extremely hard'. When a Borg scale of ∼19 (extremely hard) was reported, a cuff on the upper arm was inflated to 250 mmHg before the subject stopped grip. The PECO was employed to isolate the metaboreflex, and to examine the contribution of ATP, via the activation of P2 receptors, to the MSNA activation. One blood sample was drawn during the first minute of PECO. During the 4 min PECO, EOW was performed for 1.5 min, which started at 1 min (4 subjects) or 2.5 min (6 subjects) from the onset of cuff inflation. Passive stretch was employed to determine the contribution of ATP via activation of P2 receptors to the sensitization of mechanoreceptors under these conditions. The presented PECO data were obtained in a 1.5 min window, which started at 2.5 min or 1 min from the onset of cuff inflation. The first minute's data from PECO was not included. Subjects did not complain of any pain caused by EOW during PECO.

Local infusion of pyridoxine. After 10–15 min of recovery from the pre-infusion trial, a modified Bier block procedure was utilized to regionally administer pyridoxine into the forearm. In order to 'drain' the forearm vasculature, the arm was elevated. The arm was fitted with occlusion cuffs arranged in a continuous fashion from the wrist to the elbow (3–4 cuffs). A final cuff was placed on the upper arm. From the wrist to the upper arm, the cuffs were inflated to 250 mmHg in sequence. Then, the cuffs on the forearm were deflated and removed, and the upper arm cuff remained inflated. Thereafter, 100, 150 or 200 mg pyridoxine hydrochloride (100 mg ml^{-1}, American Pharmaceutical Partners, Inc., Schaumburg, IL, USA) in 40 ml of saline was infused into the occluded arm via an intravenous catheter that had been placed in the antecubital fossa. This allows the distribution of the drug through the previously emptied vascular system and its diffusion into the forearm tissue. The dose of pyridoxine was determined from pilot studies and the forearm volume. Based on whether the forearm volume was $<$900 ml, 900–1200 ml, or $>$1200 ml, 100, 150 or 200 mg pyridoxine was given, respectively. After 20 min, the cuff was deflated. The subjects rested for at least 15–20 min and until heart rate and blood pressure returned to baseline.

Pyridoxine trial. Another 6 min of baseline data were collected, and a blood sample was drawn. The exercise protocol was then repeated as described for the

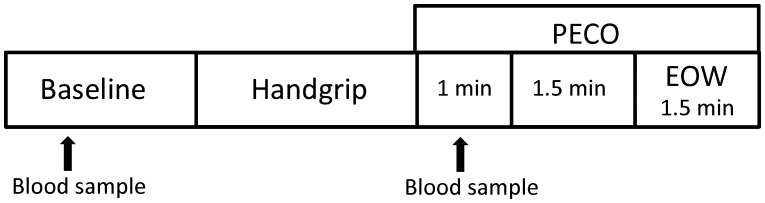

Figure 1. Experimental protocol employed in the present study
During the 4 min post-exercise circulatory occlusion (PECO), passive stretch (EOW) was performed for 1.5 min, which started at 1 min or 2.5 min from the onset of cuff inflation. The presented PECO data were obtained in a 1.5 min window, which started at 2.5 min or 1 min from the onset of cuff inflation.

pre-pyridoxine trial. A blood sample was drawn during the first minute of PECO.

To separate the effects of P2 receptor blockade from the Bier block procedure *per se*, a saline infusion study was performed in all subjects on another visit to the laboratory. The saline infusion study and the pyridoxine infusion study were performed in a random sequence. The two studies were separated by approximately one month. Cardiovascular variables and MSNA were recorded in the same fashion during the two visits. The *pre-saline trial* was the same as the *pre-pyridoxine trial*. During the local infusion procedure, 40 ml saline was infused into the arm. The handgrip exercise and PECO protocol was repeated for the *saline infusion trial*. Blood samples were not obtained during this visit.

To gain insight into whether the pyridoxine infusion would alter MSNA responses to a non-specific stimulus, a cold pressor test (CPT) was performed before and after the pyridoxine infusion in one subject (separate study day). The CPT was performed by immersing the hand (i.e. the arm with intravenous catheter) to the wrist in an ice water slurry for 2 min. The subject was instructed to remain relaxed, breathe normally and avoid Valsava-like manoeuvres during hand immersion. After 15 min of recovery from CPT, pyridoxine was infused into the forearm via the modified Bier block procedure. After 20 min recovery, the CPT was repeated.

Data analysis

Data were sampled at 200 Hz via a data acquisition system (MacLab, ADInstruments, Castle Hill, Australia). MSNA bursts were first identified in real time by visual inspection of the data, coupled with the burst sound from the audio amplifier. These bursts were further evaluated by a computer program that identified bursts based upon fixed criteria, including an appropriate latency following the R-wave of the electrocardiogram (Cui *et al.* 2006). Integrated MSNA was normalized by assigning a value of 100 to the mean amplitude of the top 10% largest bursts during the 6 min baseline period. Normalization of the MSNA signal was performed to reduce the variability between subjects attributed to factors including needle placement and signal amplification. Total MSNA was identified from the burst area of the integrated neurogram (Cui *et al.* 2006). Because in one subject the MSNA recording during the pyridoxine trial was not obtained, all MSNA recordings from this subject were removed from the statistical analysis. Mean arterial pressure (MAP) was calculated from the Finometer waveform during handgrip exercise and PECO, while the baseline MAP was verified by an automated sphygmomanometer from an upper arm. MSNA, MAP and heart rate during the last minute of handgrip were used for the data analysis of fatiguing exercise.

Statistics

Differences in the absolute values of cardiovascular variables among the baselines prior to the four exercise trials (i.e. 'pre-exercise') were evaluated via repeated measures one-way ANOVA. Differences in the absolute values of the cardiovascular variables between respective pre-exercise, the last minute of fatiguing exercise, PECO and passive stretch during PECO (PECO+EOW) were evaluated for each trial via Tukey's *post hoc* analysis after repeated measures one-way ANOVA. Changes in cardiovascular variables from the pre-exercise to the last minute of fatiguing exercise (i.e. ΔMAP, Δheart rate and ΔMSNA) were used to examine two main effects: (1) the effect of the Bier block procedure (Factor 1: before *vs.* after); and (2) the effects of drugs (Factor 2: pyridoxine *vs.* saline) via repeated measures two-way ANOVA. When appropriate, Tukey's *post hoc* analyses were employed. In a similar manner, changes from pre-exercise to PECO, and the changes from pre-exercise to passive stretch during PECO were compared 'across' Bier block and the drugs, respectively. The difference in MSNA response to EOW (changes from PECO-only to PECO+EOW) before and after pyridoxine was evaluated using Student's *t* test. Differences in the PLP levels in the blood sample obtained during the pre-pyridoxine baseline, pre-pyridoxine PECO, pyridoxine baseline and pyridoxine PECO were evaluated via repeated measures one-way ANOVA. All values are reported as means ± S.E.M. P values of <0.05 were considered statistically significant.

Results

Plasma PLP levels, the P2-purinoceptor antagonist, during the pre-pyridoxine trial and the pyridoxine trial are shown in Fig. 2. After the pyridoxine infusion, PLP during pre-exercise and PLP during PECO were significantly greater than the pre-exercise value seen before the infusion. In the pre-pyridoxine trial, the handgrip exercise itself did

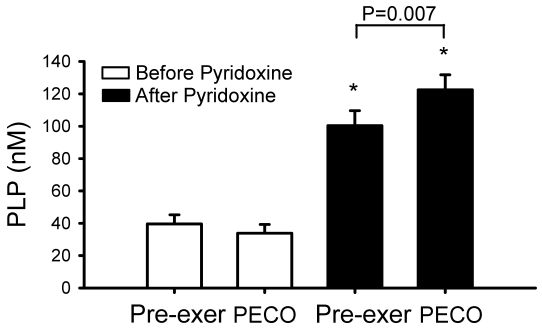

Figure 2. Plasma pyridoxal-5-phosphate (PLP) in the treated arm before and after pyridoxine infusion, during pre-exercise (Pre-exer) and PECO
*$P < 0.001$ *vs.* the pre-exercise before pyridoxine.

Table 1. Pre-exercise cardiovascular variables and MSNA before and after administration of pyridoxine or saline

	Before pyridoxine	After pyridoxine	Before saline	After saline	*P*
SBP (mmHg)	121 ± 3	125 ± 3	124 ± 3	127 ± 3	0.21
DBP (mmHg)	62 ± 1	63 ± 2	63 ± 1	63 ± 2	0.67
MAP (mmHg)	82 ± 2	84 ± 2	83 ± 1	85 ± 2	0.30
Heart rate (beats min^{-1})	59.8 ± 2.4	60.1 ± 2.3	60.3 ± 2.5	58.6 ± 2.0	0.88
MSNA (bursts min^{-1})	10.4 ± 1.4	11.5 ± 1.0	10.9 ± 1.5	10.4 ± 1.5	0.94
MSNA (units min^{-1})	129 ± 15	152 ± 18	169 ± 27	160 ± 30	0.43
Respiration (cycles min^{-1})	17 ± 1	17 ± 1	17 ± 1	16 ± 1	0.47

Values are mean ± S.E.M. SBP, DBP, MAP: systolic, diastolic and mean arterial blood pressures, which were measured by an automated sphygmomanometer from an upper arm. There is no significant difference in the measurements among the trials. *P*: *P* value from the one-way repeated ANOVA. $n = 9$ for MSNA; $n = 10$ for other variables.

not affect the PLP level. However, in the pyridoxine trial, PLP rose during PECO.

Baseline values for MSNA, blood pressure and heart rate obtained before the four trials did not differ (Table 1). Integrated MSNA recordings during handgrip, PECO and PECO+EOW in a representative subject are shown in Fig. 3. Isometric fatiguing handgrip evoked increases in MSNA, heart rate and MAP in the four trials (all $P < 0.001$). After the pyridoxine infusion, MSNA and MAP responses to handgrip (i.e. change from pre-exercise to the last minute of handgrip before fatigue) were significantly less than that in the pre-pyridoxine trial (Fig. 4). In contrast, the saline infusion had no significant effect on the MSNA response to handgrip. Thus, after the pyridoxine, the MSNA responses to exercise were significantly lower than those after saline (Fig. 4). Heart rate responses to the fatiguing handgrip did not differ in the different trials.

After pyridoxine, the MSNA and MAP responses to PECO-only (i.e. change from pre-exercise to PECO) were significantly less than those noted during the pre-pyridoxine trial (Fig. 5). After saline infusion, the MSNA and MAP responses to PECO were not lower than that during the pre-saline trial. Thus, the MSNA response to PECO after pyridoxine was significantly lower than that noted after saline infusion (Fig. 5). There was no significant difference in heart rate responses to PECO between the four trials.

Similar to the PECO-only condition, the MSNA and MAP responses to PECO + passive stretch (i.e. the change from the pre-exercise to PECO+EOW) were attenuated by pyridoxine (Fig. 6). Moreover, the MSNA response to PECO+EOW after pyridoxine was significantly lower than that noted after saline infusion (Fig. 6). There was no significant difference in heart rate responses to PECO+EOW between the four trials.

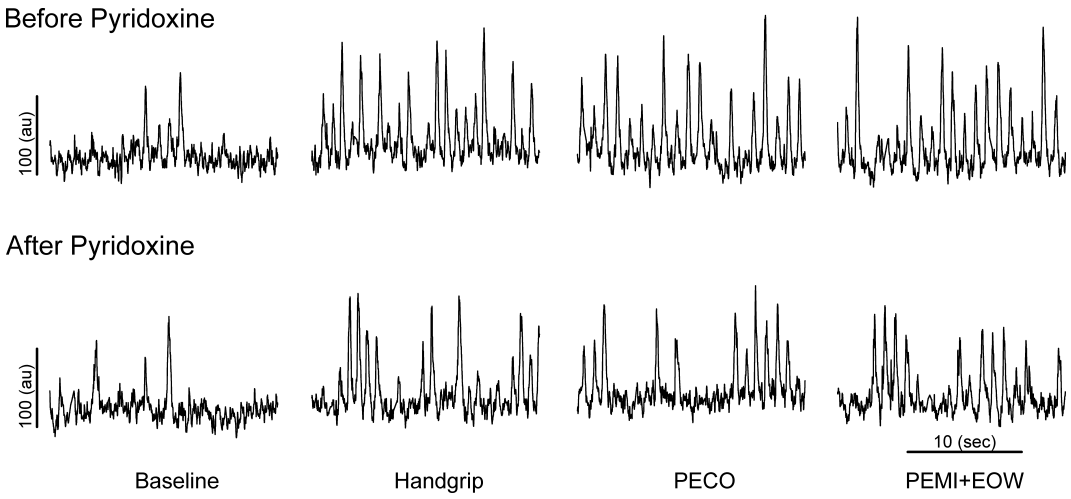

Figure 3. Representative tracing of integrated muscle sympathetic nerve activity during the isometric handgrip, PECO and EOW
Upper panel: before pyridoxine. Lower panel: after pyridoxine. au, arbitrary units.

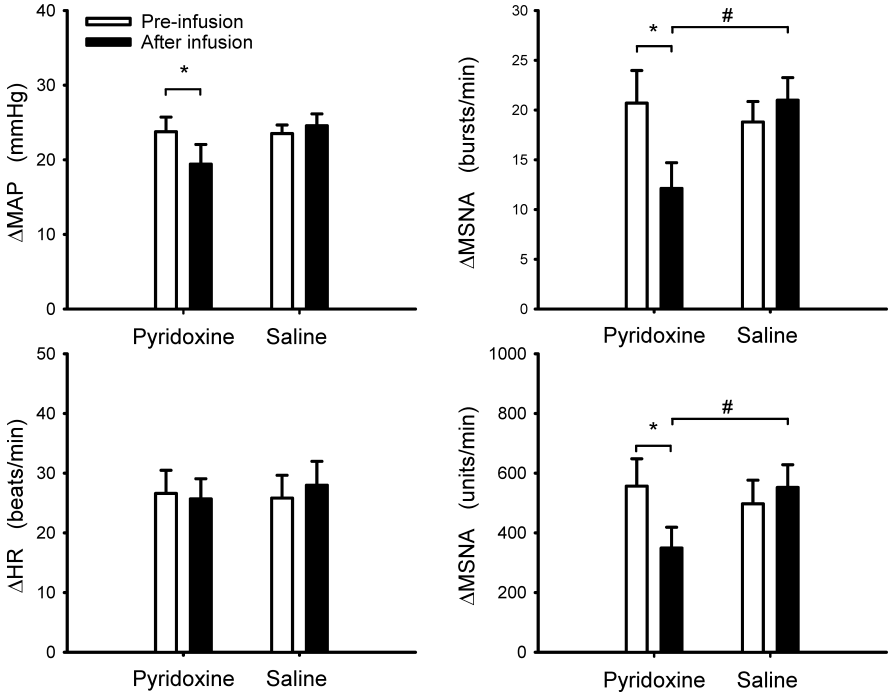

Figure 4. ΔMSNA, ΔMAP and ΔHR responses to handgrip exercise (i.e. change from pre-exercise to the last minute of fatiguing handgrip) before and after local administration of pyridoxine or saline
After the pyridoxine, MSNA and MAP responses during the last minute of handgrip before fatigue were significantly less than that in the pre-pyridoxine trial. In contrast, the MSNA response to handgrip was not statistically different between pre-saline and the saline infusion trials. $*P < 0.05$ *vs.* respective pre-infusion trial. $\#P < 0.05$ *vs.* pyridoxine trial.

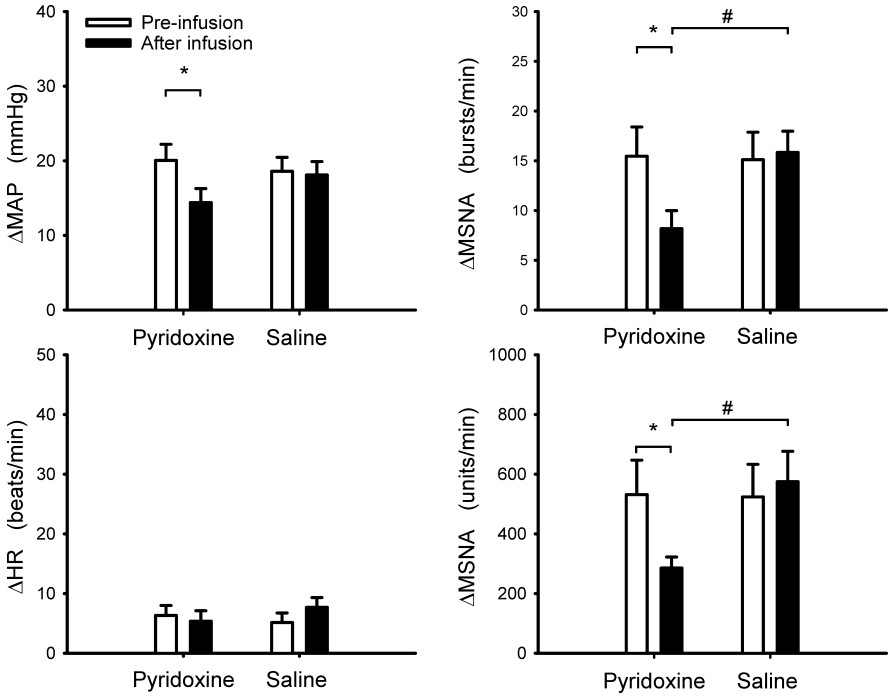

Figure 5. ΔMSNA, ΔMAP and ΔHR response to post-exercise circulatory occlusion (PECO) before and after pyridoxine or saline infusion (i.e. the change from the pre-exercise to PECO)
The data were obtained during 1.5 min PECO without passive stretch. MSNA and MAP responses to PECO after pyridoxine were significantly less than the pre-infusion trial. $*P < 0.05$ *vs.* respective pre-infusion trial. $\#P < 0.05$ *vs.* pyridoxine trial.

In the pre-pyridoxine trial, the passive stretch during PECO evoked significant increase in MSNA (25.8 ± 2.7 to 30.6 ± 3.5 bursts min^{-1}, $P < 0.01$; and 661 ± 115 to 770 ± 128 units min^{-1}, $P < 0.01$; i.e. absolute value during PECO *vs.* PECO+EOW). After pyridoxine, the passive stretch during PECO evoked a non-significant increase in MSNA (19.7 ± 2.1 to 22.8 ± 2.8 bursts min^{-1}, $P = 0.13$; and 445 ± 45 to 520 ± 78 units min^{-1}, $P = 0.11$). However, pyridoxine did not attenuate the rise in MSNA by the passive stretch (i.e. the change from PECO-only to PECO+EOW, 4.8 ± 1.3 to 3.2 ± 1.9 bursts min^{-1}, $P = 0.41$; and 109 ± 32 to 75 ± 42 units min^{-1}, $P = 0.50$).

There was no significant difference in MVC between the two visits (saline *vs.* pyroxidine: 35.4 ± 2.1 *vs.* 33.9 ± 2.0 kg, $P = 0.21$). There was no significant difference in the end-exercise Borg scale values in the four trials (before pyridoxine: 19.3 ± 0.2; after pyridoxine: 19.1 ± 0.1; before saline: 19.3 ± 0.2; after saline: 18.8 ± 0.2; two-way ANOVA; $P = 0.28$ for Bier block; $P = 0.39$ for the drugs; $P = 0.73$ for the interaction). The exercise duration was shortened similarly by local saline and pyridoxine infusions (two-way ANOVA; $P = 0.03$ for the Bier block procedure; $P = 0.45$ for the drugs; $P = 0.37$ for the interaction). After pyridoxine infusion, the exercise duration tended to be shorter than before pyridoxine (237 ± 25 to 188 ± 18 s, $P = 0.07$). After the saline infusion, the exercise duration was significantly shorter than that before saline (261 ± 19 to 193 ± 16 s, $P = 0.02$). There was no significant difference in the exercise duration between the trial after pyridoxine and the trial after saline ($P = 0.82$).

Heart rate, integrated MSNA and blood pressure recordings during CPT in the one subject are shown in Fig. 7. Pyridoxine did not alter MSNA or blood pressure responses to CPT.

Discussion

The main findings of this study are that local administration of pyridoxine increased pyridoxal-5-phosphate concentration in the human forearm, and was associated with attenuated MSNA and blood pressure responses to fatiguing exercise and the post-exercise circulatory occlusion. These results verified our hypothesis that P2 receptor antagonism in the exercising muscles attenuates sympathetic activation during fatiguing exercise. Thus, these data are consistent with the concept that the activation of P2 receptors is involved in the exercise pressor reflex.

In the present study, the exercise duration was attenuated after both pyridoxine and saline infusion. This 'order effect' has been noted previously (Seals & Enoka,

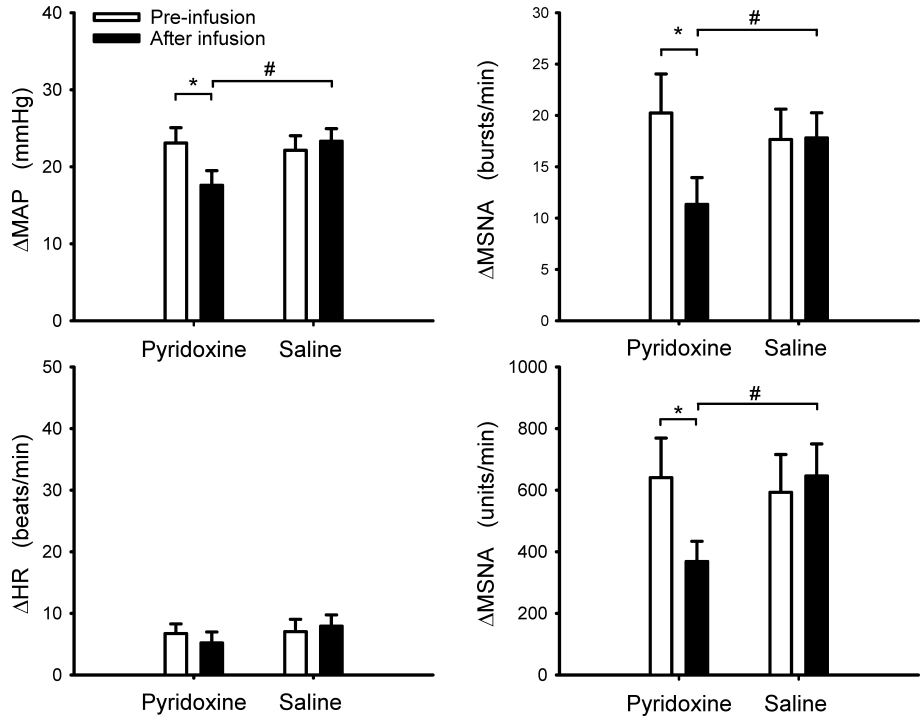

Figure 6. ΔMSNA, ΔMAP and ΔHR responses during EOW (passive stretch) in PECO conditions before and after pyridoxine or saline infusion (i.e. the change from the pre-exercise to PECO+EOW)
The EOW started at 2.5 min or 1 min from the onset of cuff inflation. The MSNA and MAP changes from pre-exercise after pyridoxine were significantly less than the pre-infusion trial. *$P < 0.05$ *vs.* respective pre-infusion trial. #$P < 0.05$ *vs.* pyridoxine trial.

1989). Of note, the exercise duration after the saline and pyridoxine infusion were similar, yet MSNA responses were less after pyridoxine than after saline. There was no significant difference in the end-exercise Borg scale values (i.e. perceived exertion) in the four trials. Moreover, there was no difference in the heart rate responses among the trials. These suggest that central command input was similar during the different trials (Williamson *et al.* 1995). We believe these findings strongly suggest that pyridoxine evoked its effect not through altered central command but through a peripheral mechanism.

P2 receptors mediate the actions of ATP and related substances. Based on differences in molecular structure and signal transduction mechanisms, P2 receptors can be differentiated into two families of ligand-gated ion channels and G protein-coupled receptors termed P2X and P2Y receptors, respectively (Ralevic & Burnstock, 1998). Both P2X and P2Y receptors are found on sensory neurons (Kennedy & Leff, 1995; Burnstock, 1996; Nakamura & Strittmatter, 1996; Cook *et al.* 1997; Harden *et al.* 1998; Humphrey *et al.* 1998). P2 receptor activation can evoke nerve impulse generation, as well as lead to the release of sensory neurotransmitters at both central and peripheral ends of afferent fibres (Kennedy & Leff, 1995; Burnstock, 1996). In the present study, pyridoxine hydrochloride was administered into the forearm. The pyridoxine was converted into PLP (Solomon & Hillman, 1979; Jansonius, 1998), a non-specific P2-purinoceptor antagonist (Khakh *et al.* 1995; Ralevic & Burnstock, 1998).

It is interesting to note that after the infusion of pyridoxine, the plasma PLP level during the PECO (occlusion) was higher than pre-exercise (freely perfused). Since handgrip before pyridoxine did not affect PLP, the most likely explanation is that the forearm circulatory occlusion after the pyridoxine infusion led to a redistribution of PLP from interstitial or intracellular sources to the bloodstream.

Intravenous infusions of ATP are not available for human use in the USA, thus we could not determine the extent of P2 receptor blockade. Nevertheless, we did note that the pyridoxine infusion raised forearm PLP levels ∼2- to 3-fold. Moreover, a previous animal study

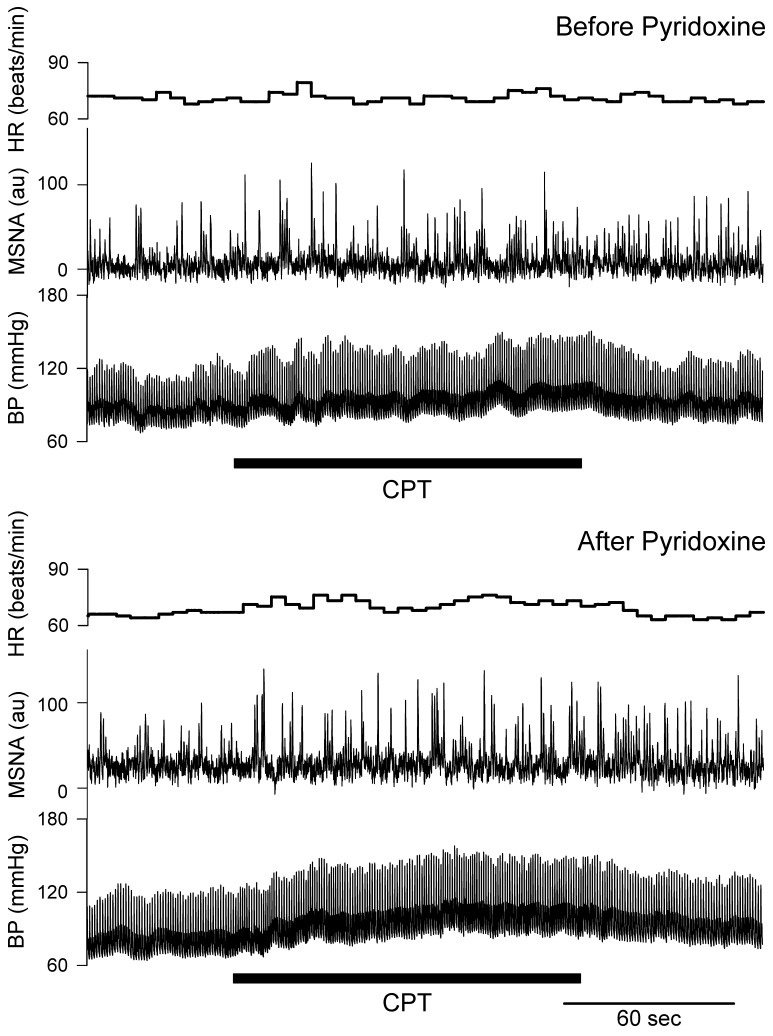

Figure 7. Heart rate, integrated MSNA and blood pressure recordings during the cold pressor test (CPT) in one subject
Upper panel: before pyridoxine. Lower panel: after pyridoxine. After pyridoxine infusion, the CPT-induced increases in MSNA and blood pressure were not less than those before pyridoxine infusion.

(Millart *et al.* 2009) demonstrated that 0.05 μM PLP (i.e. 50 nM) acted as a P2 antagonist. The measured plasma PLP after pyridoxine infusion in the present study was greater than 50 nM. Thus, we believe that the PLP concentration achieved exceeded those necessary to evoke a P2 receptor antagonism.

Animal (Li *et al.* 2003) and human (Mortensen *et al.* 2009) studies suggest that muscle interstitial ATP concentrations increase with muscle contraction, and that ATP and ATP analogues stimulate muscle afferents (Hanna *et al.* 2002; Li & Sinoway, 2002). The precise stimulus leading to a rise in ATP is not clear although mechanical stimulation of both epithelial and neuronal cells can stimulate ATP release (Vizi *et al.* 1992; Grygorczyk & Hanrahan, 1997; Watt *et al.* 1998). We postulate that mechanical stimulation of muscle is the main source of ATP (Li *et al.* 2005). It could be noted that sympathetic nerve terminals release ATP and noradrenaline (von Kügelgen & Starke, 1991; Vizi *et al.* 1992). Thus, the increase in sympathetic nerve activity seen with exercise could also contribute to the rise in muscle ATP levels. However, a previous study has demonstrated that sympathetic and motor nerves are not the major sources of ATP release in skeletal muscle. Muscle contraction *per se* appears to be the necessary and sufficient stimulus to raise ATP (Li *et al.* 2003).

In the present study, since pyridoxine attenuated the MSNA responses to exercise, but did not affect MSNA response to a CPT ($n = 1$), we suspect that the observed responses were in fact due to the P2 receptor blockade in skeletal muscles. However, we cannot exclude the possibility that pyridoxine may have effects on other processes and systems. Animal studies have demonstrated that ATP contributes to the exercise pressor responses via stimulating P2X and not P2Y receptors (Li & Sinoway, 2002; Hanna & Kaufman, 2003). We suggest that ATP in humans also stimulates P2X receptors, although this cannot be definitively proven.

When ATP or its analogue is infused into the cat hindlimb, the pressor response evoked by muscle stretch is augmented in cats (Li & Sinoway, 2002). Thus, it has been suggested that ATP stimulates and sensitizes skeletal muscle afferents (Li & Sinoway, 2002). However, in the present study, pyridoxine did not affect the MSNA response to muscle stretch. Why the present observation differs from those in the previous study (Li & Sinoway, 2002) is not clear but could be due to the difference in study design or in the models used. In the present study, circulatory occlusion was needed to bring out effects of the muscle stretch. Under these conditions, other muscle metabolites such as prostaglandins (Cui *et al.* 2008) may sensitize muscle mechanoreceptors independently of any effects on P2 receptors. For example, cyclo-oxygenase products stimulate the muscle afferents and play a role both in sensitizing group III and IV afferents during exercise (Rotto *et al.* 1990*a*,*b*; Hayes *et al.* 2006). Thus, we cannot exclude the possibility that P2 receptor activation may contribute to muscle afferent sensitization. This can be examined with low-level repeated exercise protocols, in which signal-averaging techniques (Herr *et al.* 1999; Cui *et al.* 2006) would be used to accurately determine the onset latency from the initiation of contraction to the onset of sympathoexcitation.

Study limitations

The half-life of pyridoxine is 15–20 days and the pyridoxine trial was always performed after the pre-infusion trial. To examine the influence of trial sequence, a *saline infusion* trial was performed on a separate day. PLP is a non-specific P2 receptor antagonist. Thus, the subtype of the P2 receptors engaged cannot be determined. Based on animal work (Hanna *et al.* 2002; Li & Sinoway, 2002), we suspect that PLP is evoking its effect by blocking P2X receptors.

In conclusion, the present results show that blood pressure and MSNA responses to exercise are attenuated after pyridoxine infusion. These observations are consistent with the concept that P2 receptors contribute to the exercise pressor reflex in humans.

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J.C.: conception and design of the experiments; collection, analysis and interpretation of data; and drafting the article or revising it critically for important intellectual content. U.A.L.: collection and interpretation of data. C.B.: data collection. N.C.K.: data collection and analysis. L.I.S.: conception of the experiments; drafting the article and revising it critically for important intellectual content. All authors approved the final version of the manuscript.

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